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Differential expression and seasonal variation on aquaporins 1 and 9 in the male genital system of big fruit-eating bat *Artibeus lituratus*

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ABSTRACT

Efferent ductules and epididymis are involved in water and solute transport, which is indispensable for storage and maintenance of the sperm viability. The reabsorption process involves proteins such as aquaporins (AQP), which has been described in the male genital system of limited species, including primate, rodents, cats and dogs. To contribute with information about AQPs in the male system, here we investigated the distribution of AQP1 and AQP9 in the tropical bat *Artibeus lituratus*, along the annual reproductive cycle. *A. lituratus* is a seasonal breeder with natural variation in components of the androgen and estrogen responsive system, thus being a good model for exploring the AQPs modulation. AQP1 was found restricted to differentiating spermatids, efferent ductules epithelium and venular endothelia along the male tract. AQP9 was detected throughout the epididymis being more abundant in the cauda and ductus deferens, but was not found in testis, rete testis and efferent ductules. Contrasting with AQP1 which appear to be constitutively expressed, there was seasonal variation in AQP9 expression, which was reduced in regressed epididymis. The AQP9 does not appear to be modulated by estradiol or androgens, but possibly by other factor related to luminal sperm. The establishment of specific function for aquaporins in the male tract remains undetermined; however, the cellular distribution presently found are compatible with the main function of AQP1, as a selective water channel, and AQP9, which is a conduct for water and a plethora of neutral solutes present in the epididymis milieu such as glycerol and urea.

1. Introduction

The efferent ductules and epididymis epithelia are involved in water and solute transport, which is indispensable for transport, maturation, storage and maintenance of the sperm viability in the epididymal lumen (Clulow et al., 1998; Hess, 2002; Joseph et al., 2011).The reabsorption process involves key proteins such as Na^+, K^+ ATPase, at the basolateral membrane, the apically located sodium/hydrogen exchanger (NHE3), and the cytoplasmic carbonic anhydrase II (CAII), that catalyzes the H^+ generation (Clulow et al., 1998; Hansen et al., 1999; Pastor-Soler et al., 2005). The transcellular fluid movement depends on aquaporins (AQP), which constitute a family of 13 integral membrane proteins named AQP0–AQP12 (Verkman, 2005). There are some aquaporins that selectively permeate water (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8), whereas other can transport water and other small polar molecules, mainly glycerol and urea, thus been denominated aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) (Agre et al., 1993; Cerda and Finn, 2010; Tsukaguchi et al., 1998).

Among aquaporins, the isoforms AQP1 and AQP9 have been described in the efferent ductules and/or epididymis (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Da Silva et al., 2006b; Danyu et al., 2008; Domeniconi et al., 2007, 2008; Elkjaer et al., 2000; Fisher et al., 1998; Hashem, 2010; Lu et al., 2008; Oliveira et al., 2005; Pastor-Soler et al., 2001; Rojek et al., 2007). The pattern of expression of these aquaporins varies in a cell-, region-, and species-specific manner, as well as depending on developmental phase. There is evidence that AQP9 expression in the rat efferent ductules is modulated by estrogen and dihydrotestosterone (DHT), but only by DHT in the initial segment of the epididymis (Oliveira et al., 2005). Others have shown that AQP9 is testosterone-modulated in the cauda epididymis (Pastor-Soler et al., 2002, 2010). Conversely, AQP1 has been described restricted to the efferent ductules, where it appears to be constitutively expressed (Arrighi et al., 2010a; Badran and Hermo, 2002; Fisher et al., 1998; Oliveira et al., 2005).

Despite localizations of several AQPs in different regions of the male reproductive system of primates (marmoset and man), rodents (mice and rat) and some domestic animals (cat and dog), information about aquaporins in other mammals, including Chiroptera, has not been described in the male genital system. Several interesting reproductive features have been described for

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chiropteran, including prolonged storage of sperm in the epididymis for up to ten months (Bernard, 1984). The mechanism for maintaining the sperm viability for long periods of time includes the establishment of an excessively hyperosmolar environment, as seen in the hibernating bats or even in some species of non-hibernating tropical bats (Cervantes et al., 2008; Crichton et al., 1993, 1994; Oliveira and Oliveira, 2011). Osmolalities of bat epididymal fluid may reach values as high as 1000 mOsm/L, which is much higher than other mammals (300 mOsm/L) Crichton et al., 1994; Hinton et al., 1981; Johnson and Howards, 1977; Turner, 2002. The hyperosmolar lumen dehydrates the sperm, reducing their metabolic rate, inducing quiescence (Crichton et al., 1994). Despite this peculiarity, little is known about the molecular mechanism regulating the luminal microenvironment in the male genital tract of bats.

Therefore, in this study we aim to contribute with information about the cell distribution of AQP1 and AQP9 along the testis and the male genital tract of the tropical bat *Artibeus lituratus*, comparing the reproductive and regressive periods of the annual reproductive cycle. AQP1 and AQP9 are the subtypes more consistently described in the male genital system. Furthermore, besides their diverse functions as aquaporin or aquagliceroporin, respectively, they also appear to be differentially modulated in the male genital tract (Oliveira et al., 2005; Pastor-Soler et al., 2002, 2010; Picciarelli-Lima et al., 2006). *A. lituratus* has been shown to be a seasonal breeder with natural variation in components of the androgen and estrogen responsive system in the male reproductive organs. During the regressive period, *A. lituratus* showed increased expression of estrogen receptor ERβ and androgen receptor in the testis, as well as ERα in the male genital tract, indicating an important role for these steroids in regulation of the annual reproductive cycle of this species (Oliveira et al., 2009, 2012; Oliveira and Oliveira, 2011). This physiological variation in the sex steroids responses without interference in other physiological systems, as occurs in most experimental models, such as knockouts, castrated or chemically treated models, points out that this species may be a good experimental model for exploring the distribution and modulation of AQPs in the male reproductive system.

2. Materials and methods

Adult male bats of the *A. lituratus* species were captured during the reproductive (August to beginning of December) and regressive (middle of December to early April) periods. The captures were carried out in Belo Horizonte (19° 55′S and 43° 56′W) in southeastern Brazil. The captures were performed using mist nets (3 × 12 m) that were placed on trails of flight pathways to intercept bats flying 1–2 m above the ground.

The captured males were considered adults according to parameters routinely used for determining the age of bats, such as teeth wear and ossification of the metacarpal epiphyses (Oliveira et al., 2009). The captures were authorized by the Brazilian Institute of Natural Environment and Renewable Resources (IBAMA, Brazil). The experimental procedure was approved by the Experimental Animal Ethics Committee of the Federal University of Minas Gerais (CETEA/UFMG).

2.1. Tissue preparation

The bats were weighed and anesthetized with an association of 30 mg/kg pentobarbital and 20 mg/kg ketamine chloridrate (i.p.). The perfusion was performed transcardially via the left ventricle with 10% neutral buffered formalin (NBF) for the immunohistochemical assay. After fixation, the efferent ductules and epididymis were removed and weighed together.

2.2. Immunohistochemistry

Fragments of testis, efferent ductules, epididymis and ductus deferens NBF-fixed and embedded in paraffin ($n = 5$) were used to detect the occurrence and cell distribution of AQP1 and AQP9 by immunohistochemistry. For this purpose, the sections were deparaffinized, hydrated and immersed in a 0.6% methanol hydrogen peroxide solution for blocking the endogenous peroxidase. After microwaving for antigen retrieval, normal goat serum (10%) was used for blocking nonspecific antibody binding. Then, the sections were incubated overnight at 4 °C with AQP1 or AQP9 polyclonal rabbit anti-rat primary antibody (Alpha Diagnostic International, San Antonio, TX, USA), diluted 1:300 for AQP1 or 1:500 for AQP9. After, the sections were exposed for 1 h to biotinylated secondary goat anti-rabbit antibody (Dako, Carpinteria, USA). The sections were incubated with the avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) for 30 min, and the immunoreaction was visualized using 3,3 diaminobenzidine containing 0.01% hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.6. Mayer hematoxylin was used to counterstain the sections. The immunostaining was performed in triplicate to confirm the results.

2.3. Morphometry

The immunostaining intensity for AQP1 and AQP9 was quantified by computer-assisted image analysis, based on previously reported protocols (Picciarelli-Lima et al., 2006). Pictures from 10 different areas of the efferent ductules epithelium (for AQP1 analysis) and epididymis (for AQP9 analysis) of five animals at each periods analyzed were taken by using an x40 objective lens of a Nikon Eclipse E600 microscope (Nikon Corp., Melville, USA). Digital images were processed with Adobe Photoshop CS3 (Adobe Systems, Mountain View, USA), converted to the grayscale mode and inverted. The images were then exported to Image-Tool software 3.00 (University of Texas Health Sciences Center, San Antonio, USA), for quantitative analysis. For this purpose, the stained apical areas of efferent ductules (for AQP1) and epididymis (for AQP9) were traced and measured by pixel intensity. Background intensity was determined by tracing an unlabeled area adjacent to the measured cells and subtracted from values detected in the labeled regions. Data were expressed as mean ± standard deviation.

2.4. Western blotting

Western blot assays were performed on the efferent ductules and epididymis (caput, corpus and cauda regions) to validate the use of the antibodies in bats and to confirm the immunohistochemistry results. For further confirmation of the specificity of the antibody produced in rats for use in bats, efferent ductules and epididymis of rats were previously run in parallel to the bat tissues. The assays revealed major bands of 28 and 31 kDa, in both

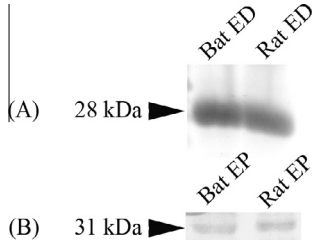

Fig. 1. Western blotting assays confirming the specificity of the antibodies for AQP1 (A) and AQP9 (B).The respective molecular weights are shown on the left. ED = efferent ductules; EP = epididymis.

tissues from bats and rats (Fig. 1). These molecular weights are in agreement with those previously found for AQP1 and AQP9, respectively, in other mammalian species (Da Silva et al., 2006b; Domeniconi et al., 2007, Elkjaer et al., 2000; Fisher et al., 1998; Lu et al., 2008; Pastor-Soler et al., 2001). These results indicate that the anti-rat AQP1 and AQP9 antibodies may cross-react with the bat proteins, thus corroborating previous findings that show that aquaporin sequences are highly conservative among mammals (Anthony et al., 2000; Higa et al., 2000; Jin et al., 2006; Kuriyama et al., 2002; Tsukaguchi et al., 1999; Wang et al., 2005; Wintour et al., 1998).

For the Western blot assays, pooled tissues of six animals in the reproductive and six animals in the regressive periods were used. The efferent ductules and epididymis tissues were macerated with dry ice, and submitted to total protein extraction and quantification by the Bradford methodology. The proteins (15 μg/lane) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes and blocked with 10% normal goat serum (NGS) for 1 h at room temperature. The membranes were incubated overnight with primary antibodies diluted 1:500 for rabbit anti-rat AQP1 or AQP9 (Alpha Diagnostic International, San Antonio, TX, USA). Then, the membranes were washed with PBS-Tween 0.05% followed by incubation with the secondary antibodies goat anti-rabbit (Dako, Carpinteria, CA, USA) diluted 1:1000. After several washes in PBS-Tween 0.05%, the reaction was developed by the addition of 0.1% of 3,3 diaminobenzidine and 0.05% of chloronaphthol in PBS, 16.6% methanol and 0.04% H_2O_2. Deionized water was used to stop the reaction. All Western blots were replicated to confirm the results.

2.5. Enzime linked immune sorbent assay – ELISA

The dosage of estradiol, testosterone and DHT in the plasma, testis and epididymis (corpus and cauda) tissue was performed by using commercial ELISA kits. For this purpose, pooled frozen testes of four animals in the reproductive and four animals in the regressive periods were macerated in dry ice. Caput of bat epididymis, including efferent ductules, are very tiny segments, therefore they could not be included in the assay due to shortage of tissue available. The samples of tissue (150 mg) were suspended in 250 μl of PBS (pH 7.4) and homogenized. After this step, lipid extraction and enrichment was performed by using diethyl ether. For this purpose, 670 μl of diethyl ether was added and subsequently vortexed. Then, the samples were centrifuged and the ether supernatant was frozen in liquid nitrogen and decanted. Afterwards, the extracts were evaporated at room temperature to about 100 μl and sonicated for 10 min. The ether was evaporated to about 20 μl, and then 60 μl of PBS was added (Hany et al., 1999). The plasma samples were obtained after centrifugation of total blood (4000 rpm for 10 min) in heparin-coated tubes. ELISA measurements were performed using 25 μl of samples per well according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany). The sensitivity of the assays for estradiol, testosterone and DHT was 9.714, 0.083, and 6.0 pg/mL, respectively. All samples were measured in quadruplicate and repeated in two independent assays.

2.6. Statistical analysis

Quantitative data was statistically analyzed by using the Matlab® (MathWorks, Natick, MA, USA) software. Initially, the data was subjected to the Lilliefors test, a modification of the Kolmogorov–Smirnov test, to check normality of the datasets. After, the data were analyzed by the Student's *t*-test to compare the means of two populations or the ANOVA test if comparisons were made between more than two populations. The significance level used for all the tests was $P < 0.05$.

3. Results

3.1. Testis and rete testis

Positivity for AQP1 was found restricted to differentiating spermatids of the seminiferous tubules epithelium in a stage-specific manner during reproductive period (Fig. 2A–H; Table 1). At stage I, the round spermatids were unreactive, whereas at stage II, the elongating spermatids showed slight positivity for AQP1. The immunoreactions were more intense in elongated spermatids at stages III and IV. Thereafter, the immunostaining decreased in elongated spermatids, which became unreactive at stage VIII. The regressed testes were unreactive for AQP1 (Fig. 2I–J). The Sertoli cells, myoid cells and Leydig cells, as well as the rete testis, were unreactive for AQP1, whereas testicular microvessels endothelia were positive (Fig. 2I). AQP9 was not detected in the testis (Fig. 2K–L) and rete testis (not shown) of *A. lituratus*, neither in the reproductive nor in the regressive periods.

3.2. Efferent ductules

The efferent ductules of *A. lituratus* were reactive for AQP1 but not AQP9 at both periods analyzed (Fig. 3). AQP1 positivity was restricted to the apical membrane of the epithelial nonciliated cells as well as vein endothelia (Table 1). The epithelial ciliated cells, peritubular smooth muscle cells, connective tissue cells, as well as arterial endothelia were immunonegative for AQP1. The cell distribution and intensity of AQP1 staining along the efferent ductules remained similar when regressive and reproductive periods were compared (Figs. 3 and 4). AQP9 was not detectable in any cell type of the efferent ductules, even when higher concentration of the antibody was used (1:25; 1:50 and 1:100).

3.3. Epididymis and ductus deferens

The epididymis and ductus deferens were unreactive for AQP1, except for the endothelia of veins, which presented positivity for AQP1 throughout the male genital tract of *A. lituratus*, irrespective of the period analyzed (Fig. 3E–F; Table 1).

During the reproductive period, AQP9 was detected along the microvilli of the principal cells of all epididymal regions, with a crescent gradient of intensity from the initial segment to cauda region (Figs. 4 and 5). Apical, basal, narrow, clear and halo cells were immunonegative for AQP9 (Fig. 5A, C, E, G). The ductus deferens stained as intensely as the cauda epididymis (Fig. 5I–J). The same pattern of AQP9 expression was observed in the regressed epididymis and ductus deferens; nevertheless, there was a significant reduction in the staining intensity along the epididymis (Fig. 4B and Fig. 5B, D, F, and H; Table 1).

3.4. Western blotting

The immunohistochemistry results and the specificity of the antibodies used were confirmed by Western blotting assays.

The AQP1 was equally detected in the efferent ductules at the reproductive and regressive periods (Fig. 6A). In the epididymis, a weak band for AQP1 was also detected, probably corresponding to the endothelial expression.

The Western blotting assays confirmed that AQP9 was undetectable in the efferent ductules, but present throughout the entire length of the epididymis (Fig. 6B). The strongest reaction was

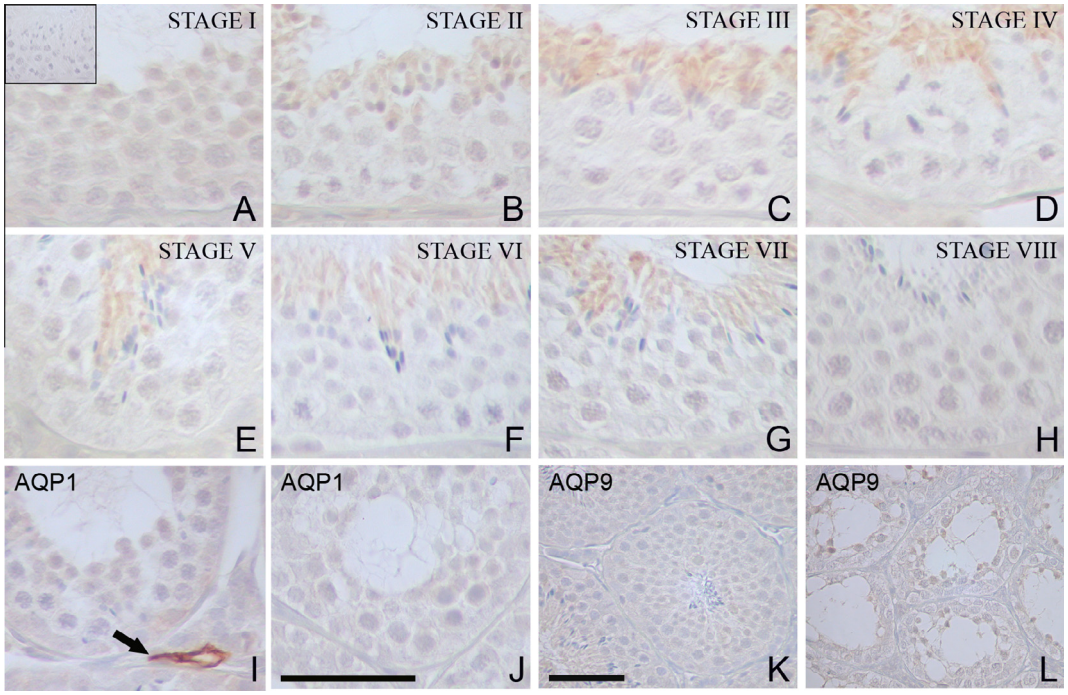

Fig. 2. Immunolocalization of AQP1 and AQP9 in the testis of *Artibeus lituratus* during the reproductive and regressive periods. (A–H) During reproductive period AQP1 was detected in differentiating spermatids of the seminiferous tubules epithelium in a stage-specific manner. (I–J) AQP1 was undetected in the regressed testis. (K–L) AQP9 was not detected in the testis in both periods analyzed. Arrow = blood vase endothelia; insert in (A) = negative control; scale bar in (J) = 50 μm, same to (A–I); scale bar in (K) = 50 μm, same to (L).

Table 1
Comparison of immunohistochemical staining for AQP1 and AQP9 in the male genital system of *Artibeus lituratus* during reproductive and regressive periods.

	AQP1		AQP9	
	Reproduction	Regression	Reproduction	Regression
Rete testis	–	–	–	–
Testis				
Leydig cells	–	–	–	–
Myoid cells	–	–	–	–
Sertoli cells	–	–	–	–
Spermatogonium	–	–	–	–
Spermatocytes	–	–	–	–
Spermatids	–/+/++	NP	–	NP
Efferent ductules				
Nonciliated cells	+++	+++	–	–
Ciliated cells	–	–	–	–
Vein endothelia	++	++	–	–
Initial Segment				
Epithelium	–	–	+	+*
Vein endothelia	++	++	–	–
Caput				
Epithelium	–	–	++	++*
Vein endothelia	++	++	––	–
Corpus				
Epithelium	–	–	+++	+++*
Vein endothelia	++	++	–	–
Cauda				
Epithelium	–	–	++++	+++*
Vein endothelia	++	++	–	–
Ductus deferens				
Epithelium	–	–	++++	+++*
Vein endothelia	++	++	–	–

– = Negative; + = weak staining; ++ = moderate staining; +/++ = weak to moderate; +++ = strong staining; ++++ = strongest staining; NP = cell not present
* Staining weaker than those at reproduction.

detected in the cauda epididymis, whereas the weakest was that of the initial segment. A significant reduction in the intensity of the AQP9 immunoreactive bands was more evident in the regressive period compared to the reproductive period.

3.5. Hormones measurement

The testosterone, DHT and estrogen levels are summarized in Table 2. The concentration of androgens was higher during the regressive period at all tissues analyzed. Higher levels of estradiol were found in the male genital system, compared to the plasma. The estradiol levels in the testis and epididymis were similar. There were no changes in the estradiol levels in both periods analyzed.

4. Discussion

This is the first description of the occurrence and distribution of aquaporins 1 and 9 in the male genital system of a bat species. *A. lituratus* showed a segment-specific distribution of AQP1 and AQP9 throughout the male reproductive system. Among the results, the occurrence of AQP1 in the testis and the absence of AQP9 in the efferent ductules have not been described for any species of mammals investigated to date. The AQP1 was found restricted to differentiating spermatids in the testis, nonciliated cells in the efferent ductules and venular endothelium along the male genital tract. On the other hand, AQP9 was detected throughout the epididymis and ductus deferens, but not in the testis, rete testis and efferent ductules. Contrasting with AQP1, which appears to be constitutively expressed, there was seasonal variation in the expression of AQP9. The differential distribution and regulation of both aquaporins may reflect different physiological significance for these proteins in the male genital tract.

In *A. lituratus*, AQP1 was found restricted to elongating and elongated spermatids from stage II to VII of the seminiferous epithelium cycle. These results differ from other species in which AQP1 has not been described in any cell of the testis (Fisher et al., 1998; Lu et al., 2008; Nicotina et al., 2005). Besides our

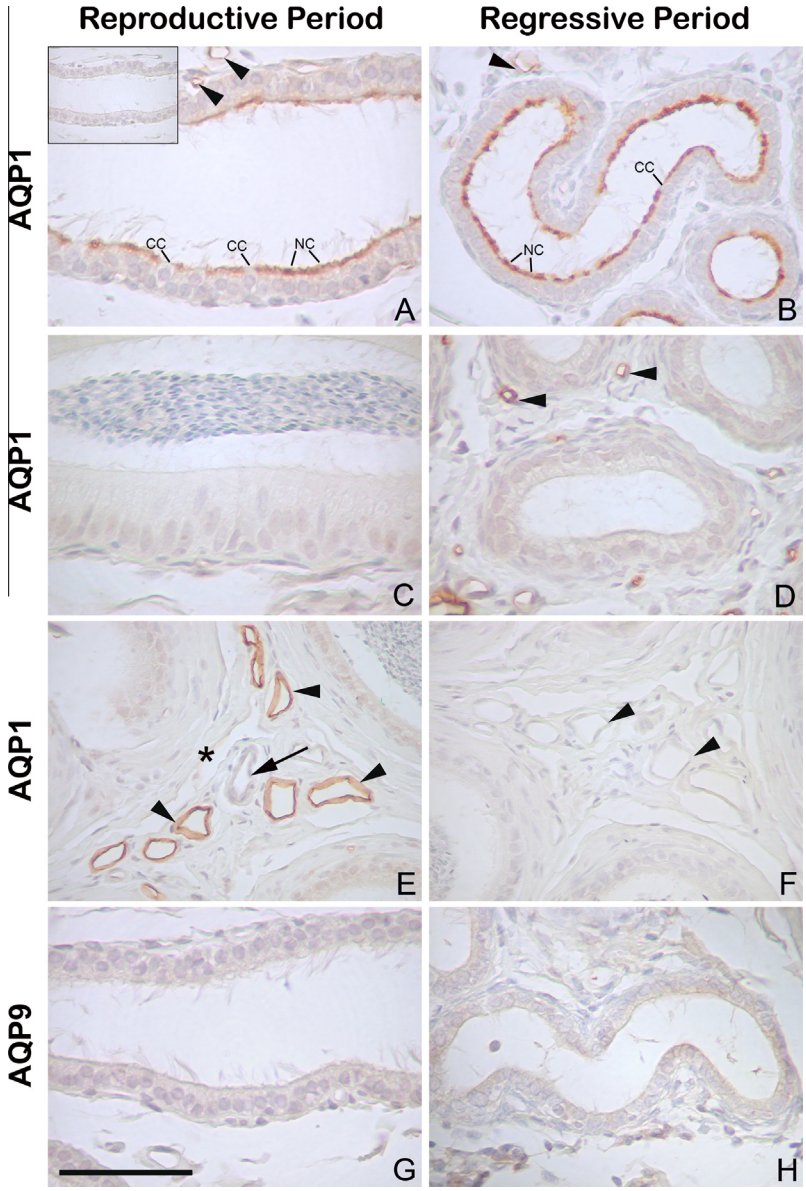

Fig. 3. Immunolocalization of AQP1 and AQP9 in the efferent ductules and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. (A–B) AQP1 positivity was detected in apical membrane of the epithelial nonciliated cells in both reproductive and regressive periods. (C–D) In the epididymis, only endothelia of veins were positive for AQP1. (E–F) In the interstitium of the male tract the endothelia of veins (arrowheads), but not arteries (arrow) and lymphatic vases (∗), were positive for AQP1. (G–H) The efferent ductules were unreactive for AQP9 in both periods analyzed. NC = nonciliated cells; CC = ciliated cells; insert in (A) and Fig. F = negative controls; scale bar in (G) = 50 μm.

present result, spermatids positive for aquaporins have been described only for rats (AQP7 and AQP8) and humans (AQP7) Calamita et al., 2001; Ishibashi et al., 1997; Suzuki-Toyota et al., 1999; Yeung et al., 2010. The reason for the discrepancy between species is not clear; however it is not uncommon that diverse aquaporins have similar function despite species-specific distribution. Therefore, the detection of diverse aquaporins in the spermatids offers evidence that they may be important for spermatozoa differentiation, possibly allowing the efflux of water necessary for cytoplasmic condensation and reduction of cell size during the spermiogenic process.

AQP9 was undetectable in the testis of *A. lituratus*. In line with our findings, other studies also failed in detecting AQP9 in the dog, mice and human testis (Domeniconi et al., 2007; Hashem, 2010; Ko et al., 1999; Tsukaguchi et al., 1999). Conversely, AQP9 has been found exclusively in the Leydig cells of rat (Badran and Hermo, 2002; Elkjaer et al., 2000; Nicchia et al., 2001; Nihei et al., 2001). We do not have a clear explanation for these differences, but they highlight the necessity of further investigation of other aquaporin subtypes in the species already studied, as well as investigation of other mammal species, before any clear conclusion about the role of aquaporin in the testis could be made.

Our findings showing AQP1 at the apical membrane of nonciliated cells of the efferent ductules epithelium corroborate the evidences that AQP1 is the only aquaporin subtype consistently found across species to date (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Brown et al., 1993; Domeniconi et al., 2007, 2008; Fisher et al., 1998; Lu et al., 2008; Oliveira et al., 2005; Ruz et al., 2006). The cell distribution of AQP1 is compatible with the reabsorptive function of the efferent ductules, which is responsible for reabsorption of more than 90% of the fluid coming from the testis (Clulow et al., 1998; Hess, 2002).

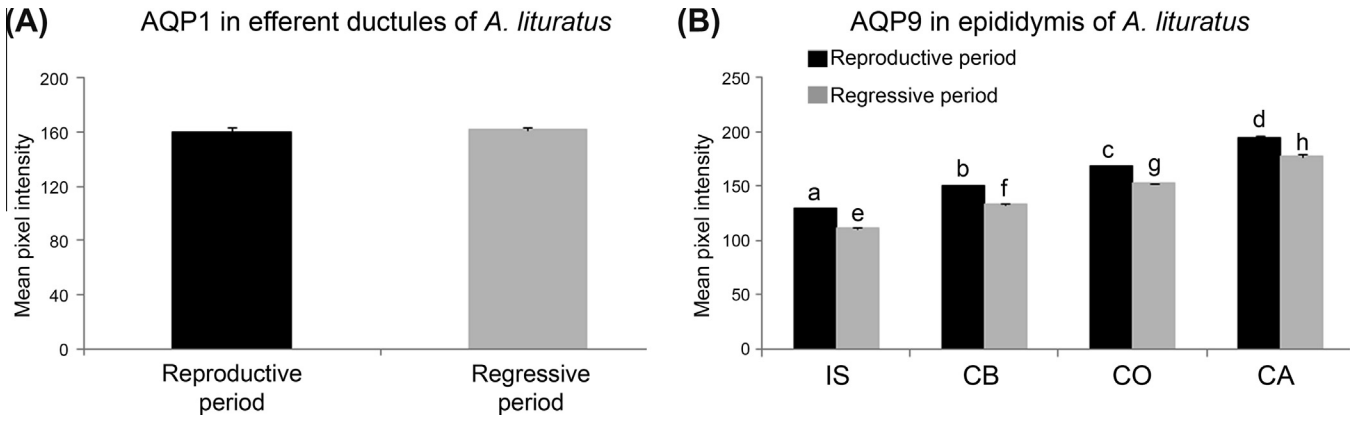

Fig. 4. Quantification of AQP1 and AQP9 staining intensity in the efferent ductules and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. (A) Immunoexpression of AQP1 in efferent ductules was similar when reproductive and regressive periods were compared. (B) The immunostaining for AQP9 was significantly increased from initial segment to cauda in both periods analyzed. During regressive period occurred a significant reduction in staining at all epididymal segments. Mean values with different letters represent significant differences ($P < 0.05$), $n = 5$.

AQP1 levels were similar when *A. lituratus* efferent ductules at reproductive and regressive periods were compared, pointing out that this protein may have constitutive expression in this segment. In fact, evidence of constitutive expression of AQP1 has been a common finding for other species and experimental models. On this sense, AQP1 was unchanged after orchidectomy, efferent ductules ligation, administration of GnRH antagonist or testosterone and dihydrotestosterone replacement, as well as undernutrition, suggesting that AQP1 regulation is not affected by testicular factors, including androgens (Arrighi et al., 2010a; Badran and Hermo, 2002; Fisher et al., 1998; Oliveira et al., 2005). Concerning estrogens, treatment with the antiestrogen ICI 182,780 or estradiol replacement after castration also did not alter AQP1 staining in the efferent ductules epithelium (Oliveira et al., 2005). Conversely, the AQP1 levels were diminished after neonatal exposition to diethylstilbestrol or genetic inactivation of estrogen receptor ERα (αERKO); however, these alterations appear to be secondary to alterations in the epithelial cytoarchitecture (Fisher et al., 1998; Ruz et al., 2006).

The presence of AQP1 in the venular endothelia throughout the male genital system of *A. lituratus* is in agreement with previous studies (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Nicotina et al., 2005; Oliveira et al., 2005), even though the identification of the stained vessels was frequently omitted. The functional role attributed to the endothelial AQP1 has been removal of water reabsorbed from the epithelium and maintenance of the water equilibrium in the interstitial tissue (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Da Silva et al., 2006a; Nicotina et al., 2005). Differing from our results, others have found AQP1 in aorta endothelium, where they appear to be involved in nitric oxide transportation besides water (Herrera and Garvin, 2007). The expression of AQP1 in venular but not arterial or lymphatic endothelia of the male genital tract further emphasizes the heterogeneity in endothelial properties between organs and vessel types.

Concerning AQP9, contrasting with previous descriptions for rat, mice, dog and cat (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Domeniconi et al., 2007; Oliveira et al., 2005; Ruz et al., 2006), the efferent ductules of *A. lituratus* were unreactive for AQP9. It is well established that, despite the fact that AQP9 presents similar permselectivity in different species, there are distinct patterns of tissue distribution, possibly due to the different requirements of metabolites among the species (Tsukaguchi et al., 1999). On this sense, rats and bats differ in the structure of the efferent ductules, as in bats the efferent ductules are more numerous (12–15) and enter the epididymis separately through about six terminal ductules (Oliveira et al., 2012; Oliveira and Oliveira, 2011), whereas in rats all 6–8 ductules converge into one common duct that continues directly into the epididymal duct (Ilio and Hess, 1994). It is known that, as the sperm progress from proximal to distal efferent ductules there is substantial fluid reabsorption, concentrating the luminal spermatozoa (Ilio and Hess, 1994). Therefore, the anatomical difference may reflect in higher compaction of spermatozoa into the rat ductules, thus justifying the requirement of additional AQP, such as AQP9, to better establish the adequate luminal milieu for maintenance of the spermatozoa. To corroborate this point of view, information about AQP9 distribution in other species with efferent ductule morphophysiology similar to that of bats, such as humans, would be helpful. On the other side, one cannot rule out the possibility that other aquaglyceroporins, equivalent in function to AQP9, are expressed in the bat ductules.

As presently found, epididymis epithelium strongly positive for AQP9 but unreactive for AQP1 has been consensual in the literature (Badran and Hermo, 2002; Domeniconi et al., 2007, 2008; Oliveira et al., 2005). The pattern of AQP9 immunoreaction showed a gradual increase from the initial segment to the cauda epididymis of *A. lituratus*. This pattern of expression paralleled the increase in luminal osmolality from caput to cauda, which is considered an important mechanism for sperm maturation (Cooper and Yeung, 2003). Moreover, in hibernating bats, the hyperosmolar environment of cauda epididymis prolongs the survival and guarantees the viability of spermatozoa for long periods of time (Crichton et al., 1994). Thus, considering that spermatozoa concentration is gradually increased from caput to cauda, the higher levels of AQP9 may play a role in the process of maintenance of the hyperosmolar environment necessary to keep the sperm quiescence. On the other hand, it is well known that the caudal region of the epididymis is responsible for the storage and protection of spermatozoa (Hermo and Smith, 2011). As member of the aquaglyceroporin group, the AQP9 presents high permeability to glycerol, urea and other small noncharged solutes, besides water (Tsukaguchi et al., 1998), thus indicating that this protein may participate in other physiological processes beyond water homeostasis. On this sense, a possible role for AQP9 in the epididymis may be transport of glycerol, which is a known component of the luminal fluid, serving as an energy substrate for the stored sperm (Cooper and Brooks, 1981). Corroborating this point of view, functional assays have demonstrated that the epididymis is permeable to glycerol in a manner dependent of AQP9, which is in turn regulated by cAMP and bradykinin (Belleannee et al., 2009; Pietrement et al., 2008).

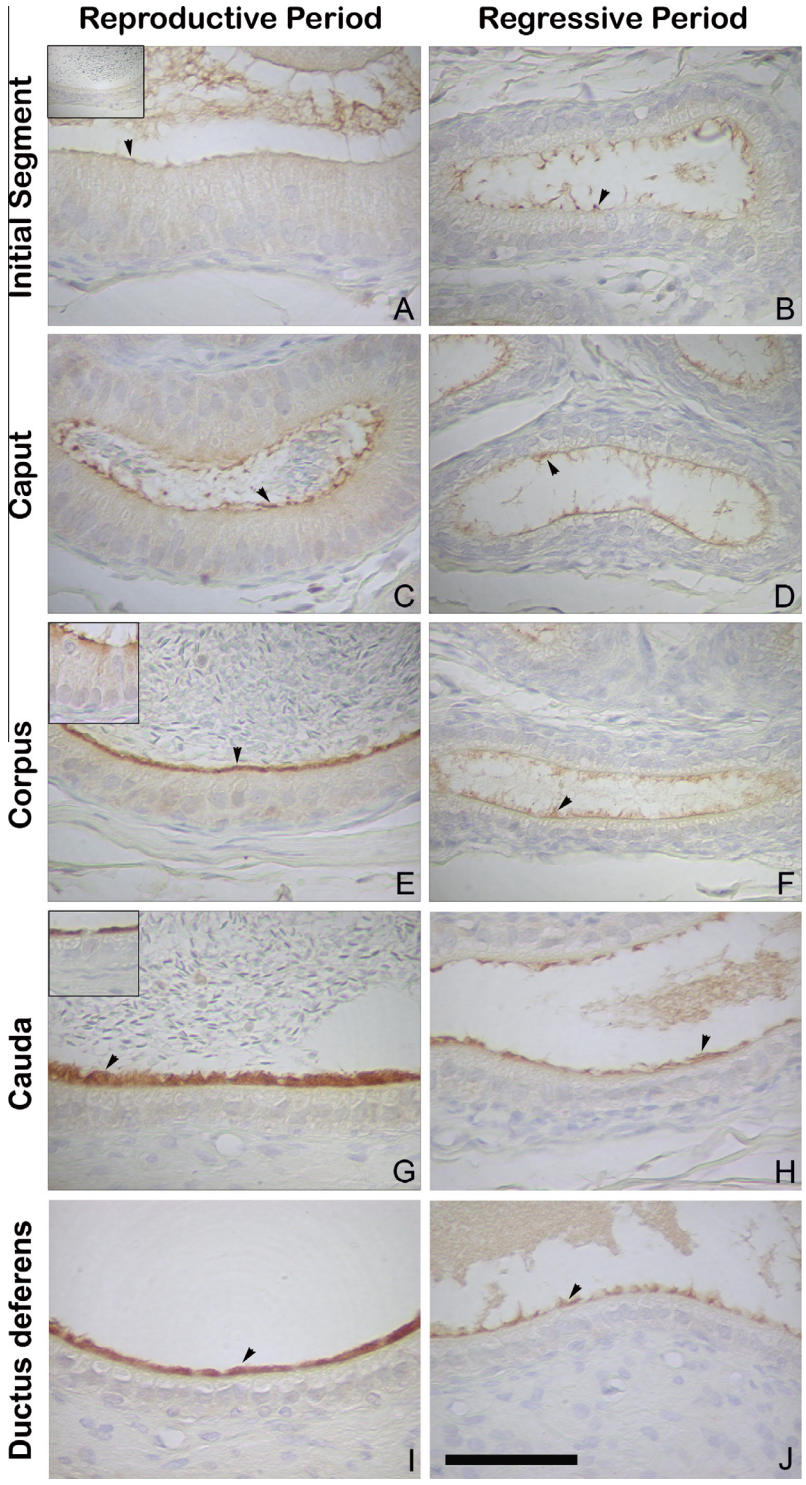

Fig. 5. Immunolocalization of AQP9 in the epididymis and ductus deferens of *Artibeus lituratus* during the reproductive and regressive periods. (A, C, E and G) During the reproductive period AQP9 was detected in the microvilli of the principal cells along the epididymis with a gradient of intensity increasing from initial segment to cauda. (B, D, F and H) During the regressive period the same pattern of AQP9 staining was observed, but in lower levels. (I–J) The ductus deferens presents similar staining intensity as the cauda. Arrowheads = positivity for AQP9; insert in (A) = negative control; Insert in (E) = negative apical and narrow cells; insert in (G) = negative clear cell; scale bar in (J) = 50 μm.

Indeed, the expression of AQP9 paralleled the luminal concentration of sperm along the *A. lituratus* epididymis (Oliveira et al., 2012). As the AQP7 has been described in sperm of several species (Calamita et al., 2001; Saito et al., 2004; Yeung et al., 2009), this aquaglyceroporin may allow the influx of glycerol coming from the luminal milieu.

Considering the broad spectrum of molecules that may permeate AQP9, it is reasonable to expect multiple functional roles for this protein. In line with this point of view, as high levels of urea have been described in cauda epididymis (Turner and Cesarini, 1983; Turner et al., 1979), another possibility is that AQP9 may be involved in urea transportation from the epididymis, thus

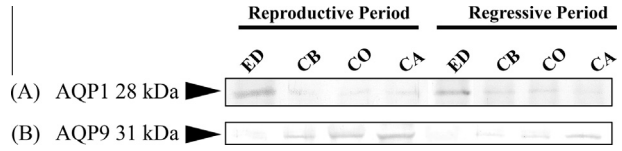

Fig. 6. Western blotting assays for AQP1 in efferent ductules (A) and AQP9 in the epididymis (B) of *Artibeus lituratus*. The respective molecular weights are shown on the left. ED = efferent ductules; CB = caput; CO = corpus; CA = cauda.

Table 2
Levels of testosterone, DHT and estradiol in the plasma, testis and epididymis of *Artibeus lituratus* during the reproductive and regressive periods.

	Reproduction	Regression
Plasma		
Testosterone (ng/mL)	0.3 ± 0.1	1.7 ± 0.5*
DHT (pg/mL)	77.0 ± 55.8	233.0 ± 171.3*
Estradiol (pg/mL)	24.0 ± 3.9	19.0 ± 2.0
Testis		
Testosterone (ng/mL)	9.0 ± 0.4	26.0 ± 0.8*
DHT (pg/mL)	2686.0 ± 308.0	4538.0 ± 786.0*
Estradiol (pg/mL)	32.0 ± 1.9	38.0 ± 7.9
Corpus epididymis		
Testosterone (ng/mL)	1.0 ± 0.1	4.1 ± 0.5*
DHT (pg/mL)	ND	ND
Estradiol (pg/mL)	33.5 ± 2.0	36.0 ± 0.1
Cauda epididymis		
Testosterone (ng/mL)	0.8 ± 0.1	1.4 ± 0.5*
DHT (pg/mL)	ND	ND
Estradiol (pg/mL)	36.2 ± 0.2	36.0 ± 0.9

DHT = Dihydrotestosterone; ND = not determined.
* $P < 0.05$.

presenting a role in detoxification of the luminal environment. Corroborating this hypothesis, there is evidence that, in hepatocytes, AQP9 indeed facilitates glycerol influx and urea efflux, being more permeable to urea and glycerol than water (Carbrey et al., 2003). Recent findings have also attributed a role for urea uptake in improving skin barrier and antimicrobial defense (Grether-Beck et al., 2012). Considering that both barrier and antimicrobial defense are important roles attributed to the cauda epididymis, it would be of interest to further investigate whether local urea uptake by AQP9 may also contribute to these local functions.

During the regressive period, AQP9 presented the same pattern of distribution in the epididymis as that observed during the reproductive period of *A. lituratus*, although with significant reduction in the protein levels. Androgens have been a factor described as a modulator of epididymal AQP9 (Oliveira et al., 2005; Pastor-Soler et al., 2002, 2010). However, here we show that AQP9 expression is reduced in the regressed epididymis, even with high levels of local, testicular and plasmatic androgens (present results), as well as androgen receptor expression (Oliveira et al., 2012). These findings corroborate previous suggestion that AQP9 is modulated by other luminal factors than androgens (Badran and Hermo, 2002). Estradiol does not appear to be a factor modulating AQP9 in the epididymis of *A. lituratus*, as the concentration of this steroid is not changed along the annual cycle, neither locally nor systemically. Lack of estradiol modulation of AQP9 in the epididymis has also been described for rat (Oliveira et al., 2005). Corroborating these data, there is evidence that the aromatase levels in the epididymis of *A. lituratus* do not vary during the annual reproductive cycle (Oliveira et al., 2012). Together, these findings add to previous suggestion that the mechanism of regulation of AQP9 in the male genital tract may be more complex than previously anticipated (Badran and Hermo, 2002; Belleannee et al., 2009; Oliveira et al., 2005; Picciarelli-Lima et al., 2006; Pietrement et al., 2008).

5. Conclusion

In summary, we have characterized for the first time the expression of AQP1 and AQP9 in the male genital system of a bat species and demonstrated that these proteins present cell- and region-specific distribution and differential seasonal variation, as AQP1 was constitutively expressed restricted to differentiating spermatids in the testis, efferent ductules nonciliated cells and venous endothelia, whereas seasonally-modulated AQP9 was detected throughout the epididymis epithelium being more abundant in the cauda and ductus deferens. The AQP9 does not appear to be modulated by estradiol or androgens, but possibly by other factors related to luminal sperm. The establishment of specific functions for aquaporins in the male genital tract remains to be elucidated; however, the cellular distribution presently found is compatible with the main function of AQP1, as a selective water channel, important for the spermiogenic process as well as for fluid reabsorption in the efferent ductules, and AQP9, which is a conduct for water and a plethora of neutral solutes present in the luminal content of the epididymis, such as glycerol and urea, among others, which may be required for the maintenance of an adequate milieu for the spermatozoa. Some differences between species were presently highlighted, indicating that further studies in diverse species will be necessary in order to allow a better interpretation of the role of aquaporins in each segment of the male genital tract.

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